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2-Deoxyglucose-6-phosphate (2-DOG-6-P) phosphatase DNA sequences as selection marker in plants

SUMMARY OF THE INVENTION

The present invention relates to the use of DNA sequences encoding a protein with the biological activity of a 2-deoxyglucose-6-phosphate (2-DOG-6-P) phosphatase as selection marker in plant cells for selecting transformed plants. Furthermore, the invention relates to recombinant DNA molecules containing such DNA sequences, with the latter being operably linked to regulatory sequences of a promoter active in plants and transcription termination and/or polyadenylation signals. Also, vectors, host cells and kits are described containing such recombinant DNA molecules, as well as plant cells and plants transformed with the recombinant DNA molecules. The present invention furthermore relates to processes for producing transgenic plants which due to the introduction of the above-described recombinant DNA molecules can be selected on media containing 2-deoxyglucose. Finally, the present invention relates to transgenic plants, plant cells and tissues containing the DNA molecule according to the invention or being obtained by the process described above, as well as harvest products and propagation material of the transgenic plants described.

move paragraph (A)

BACKGROUND OF THE INVENTION

It is possible to specifically integrate foreign genes into the plant genome by genetic engineering. This process is referred to as transformation and the resulting plants as transgenic plants. The main objectives are plant protection and an increase in quality of the harvest products. Examples of plant protection measures are: (i) herbicide-tolerant plants (DE-A-3701623; Stalker (1988) Science 242, 419), (ii) insect-resistant plants (Vaek (1987) Plant Cell 5, 159-169), (iii) virus-resistant plants (Powell (1986) Science 232, 738-743) and (vi) ozone-resistant plants (Van Camp (1994) BioTech. 12, 165-168). Examples of increase in quality are: (i) decrease in perishability of fruits (Oeller (1991) Science 254, 437-439), (ii) increase in starch production in potato tubers (Stark (1992) Science 242, 419), (iii) modification in starch (Visser (1991) Mol. Gen. Genet. 225, 289-296) and lipid composition (Voelker (1992) Science 257, 72-74) and (iv) production of polymers foreign to the plant (Poirer (1992) Science 256, 520-523).

A prerequisite for producing transgenic plants is the availability of suitable transformation systems and the existence of selectable marker allowing the identification of successfully transformed plant cells.

For the transformation there are presently several methods available. The method most frequently used for transforming dicotyledonous plants is the Agrobacterium-mediated gene transfer. Here, use is made of the natural capability of the soil bacterium of integrating genetic material into the plant genome. Further suitable methods are, e.g., protoplast transformation by polyethylene glycol induced DNA transfer, electroporation, sonication or microinjection as well as the transformation of intact cells or tissues by micro- or macroinjection into tissues or embryos, tissue electroporation, incubation of dry embryos in DNA-containing solution, vacuum infiltration of seed and the biolistic gene transfer.

Since quite independently of the method of transformation only few cells carry the desired properties, a selectable marker is integrated into the plant genome by conventional methods besides the target gene which allows the identification of transgenic cells. Presently, mainly genes are used for selecting transformed plant cells that mediate a herbicide or antibiotics tolerance. Suitable resistance genes are, e.g., the bar gene from Streptomyces hygroscopicus, which mediates resistance to the total herbicide phosphinothricine (De Block (1987) EMBO J. 6, 2513-2518), or the nptII gene from the transposon Tn5 of Escherichia coli, which confers resistance to the antibiotic kanamycin (Herrera-Estrella (1983) EMBO J. 2, 987-995). Depending on the plant species, the methods mentioned are not always effective and frequently negatively affect plant regeneration. Also, the use of genes mediating antibiotics resistances is undesired in the foodstuff sector. Furthermore, it is necessary to manipulate several enzymatic steps to control complex metabolic processes, i.e., it is essential in the area of "metabolic engineering" to allow for the possibility of multiple transformations of transgenic plants. The above-mentioned reasons have prompted the intensive search for other selectable markers. Despite intensive efforts only few new markers have been successfully used for selecting transformed plant cells. On the basis of the expression of a mannose-6-phosphate isomerase a positive selection on mannose-containing culture media for transformed plant cells could be established (WO 94/20627). Another process makes use of the capability of a deaminase from Aspergillus terreus to detoxicate the insecticide Blasticidin S (Tamura (1995) Biosci. Biotechnol. Biochem. 59, 2336-2338).

→ Insert (A)

→ Insert (B)

DETAILED DESCRIPTION OF THE INVENTION

The problem underlying the present invention is therefore to provide recombinant DNA molecules which contain a DNA sequence useful for the selection of transformed plant cells or plants.
This problem is solved by the provision of the embodiments characterized in the patent claims.

Thus, the present invention relates to a recombinant DNA molecule comprising

(a) regulatory sequences of a promoter active in plants;
(b) operably linked thereto a DNA sequence encoding a protein with the biological activity of a 2-deoxyglucose-6-phosphate (2-DOG-6-P) phosphatase; and
(c) operably linked thereto regulatory sequences which may serve as transcription termination and/or polyadenylation signals in plants.

In the context of the present invention a protein with the biological activity of a 2-DOG-6-P phosphatase is understood to be a protein which is capable of converting non-metabolizable glucose-analogous compounds such as 2-DOG into non-toxic products. 2-DOG becomes toxic after phosphorylation to 2-DOG-6-P, i.e., the phosphatase offsets the effect of 2-DOG-6-P by dephosphorylation. An alternative resistance mechanism which may also be used within the meaning of the invention is to prevent the phosphorylation or the uptake of 2-DOG. Corresponding mutants are described in yeast, e.g., a transport mutant (Novak (1990) FEBS Lett. 269, 202-204) and a phosphorylation mutant (Lobo (1977) Mol. Gen. Genet. 157, 297-300).

It was surprisingly found that the expression of a 2-DOG-6-P phosphatase from yeast can confer resistance to 2-DOG and can be used to select transformed plants which are otherwise phenotypically normal and fertile. It was known from research done on yeast that the addition of 2-DOG, a non-metabolizable glucose analogue results in the inhibition of the respiration and the growth of cells (Heredia (1964) Biochem. Biophys. Acta 86, 216). In yeast cells the growth inhibition is correlated with a reduced synthesis of structural polysaccharides (Kratky (1975) Eur. J. Biochem. 54, 459) and a blockage of the protein glycosylation (Datema & Schwarz (1978) Eur. J. Biochem. 90, 505), which presumably are caused by changes of the sugar nucleotide concentrations. Beyond the biochemical changes the addition of 2-DOG frequently results in a repression of many genes (summarized in Gancedo (1992) Eur. J. Biochem. 206,

In a preferred embodiment the DNA sequence encoding a protein with the biological activity of a 2-DOG-6-P phosphatase is selected from the group consisting of

(a) DNA sequences comprising a nucleotide sequence encoding the amino acid sequence indicated in SEQ ID NO. 2;

(b) DNA sequences comprising the nucleotide sequence indicated in SEQ ID NO. 1;

(c) DNA sequences comprising a nucleotide sequence hybridizing to a strand complementary to the nucleotide sequence of (a) or (b);

(d) DNA sequences comprising a nucleotide sequence which is degenerate to the nucleotide sequence of (c); and

(e) DNA sequences being a derivative, analogue or fragment of any nucleotide sequence of (a), (b), (c) or (d) and encoding a protein having 2-DOG-6-P phosphatase activity.

In the context of the present invention the term "hybridization" signifies hybridization under conventional hybridization conditions, preferably under stringent conditions as described for example in Sambrook (Molecular Cloning, A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

DNA sequences hybridizing to the DNA sequences encoding a protein with the biological activity of a 2-DOG-6-P phosphatase can be isolated from, e.g., genomic or cDNA libraries prepared from yeast. Such DNA sequences may be identified and isolated, e.g., by hybridization according to standard techniques (see, e.g., Sambrook, 1989, Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) using, e.g., the DNA sequences which have exactly or substantially the nucleotide sequence indicated under SEQ ID NO. 1 or parts of these sequences, or the reverse complements of these DNA sequences. The fragments used as hybridization probes may also be synthetic fragments which were prepared according to conventional synthesis techniques and the sequence of which is substantially the same as that shown in SEQ ID NO. 2. The DNA sequences encoding a protein with the biological activity of a 2-DOG-6-P phosphatase include DNA sequences the nucleotide sequences of which are degenerate to one of the above-described DNA sequences. The degeneracy of the genetic code allows the skilled person inter alia the possibility of adapting the nucleotide sequence of the DNA sequence to the codon preference of the respective host, preferably plants.

subject matter of the invention. Another subject matter of the invention are plants and plant tissues containing the above-described transgenic plant cells. Depending on the promoter chosen (e.g., 35S CaMV) the adult plants, too, are resistant to 2-DOG and may be selected for by adding this compound. When using, e.g., tissue-specific promoters this is not possible and the person skilled in the art may rely on, e.g., molecular-biological methods such as PCR in order to identify these plants. Those skilled in the art may also place seed of such plants on 2-DOG containing media after, e.g., self-fertilization or back-crossing against the parent, and may draw conclusions from the germination capacity of these seeds or the survival of the plants at a later stage of development (depending on the promoter chosen) whether the plants are transgenic or not. The transgenic plants may generally be plants of any plant species, i.e., both monocotyledonous and dicotyledonous plants. Preferably, the plants are useful plants such as wheat, barley, rice, rape, pea, maize, sugar beet, sugar cane or potato.
The invention also relates to the propagation material and harvest products of the plants according to the invention, for example, fruits, seeds, tubers, root stocks, seedlings, cuttings, etc.

As already explained above, the present invention provides recombinant DNA molecules and vectors which are suitable as selection markers in plant cells for the selection of transformed plant cells as well as transgenic plants, plant cells and/or tissues according to the invention derived therefrom. Thus, the present invention also relates to the use of a recombinant DNA molecule according to the invention or a vector according to the invention for the production of transgenic plants, plant cells and/or tissues as well as their use as selectable markers in plant cell and tissue culture and/or plant breeding.

~~The figures show:~~ BRIEF DESCRIPTION OF THE DRAWINGS

Move section (B)

Figure 1

Possible mechanisms of growth inhibition by 2-deoxyglucose (2-DOG) and removal of the toxic effect by expression of a 2-DOG-6-P phosphatase.

Figure 2

A. Cultivation of tobacco and potato leaf disks on 2-DOG-containing MS medium. TOB, Nicotiana tabacum Var. Samsun NN; POT, Solanum tuberosum var. Solara; A, E: control MS medium without addition of 2-deoxyglucose; B, F: MS medium with 0.05% 2-DOG; C, G: MS medium with 0.1% 2-DOG; D, H: MS medium with 0.5% 2-DOG.

B. Cultivation of pea, rape and wheat leaf disks on 2-DOG containing MS medium. R, rape; P, pea; W, wheat.
0.0, 0.1 and 0.5: added 2-DOG concentration in %.

Figure 3

Schematic representation of the PCR amplification of the 2-DOG-6-P phosphatase from Saccharomyces cerevisiae strain 288C

Figure 4

Plant expression cassette for the overexpression of the $DOG^{R}1$ gene from yeast (Saccharomyces cerevisiae strain S288C) in transgenic plants
Fragment A (529 bp): contains the 35S promoter of the Cauliflower Mosaic Virus (CaMV). It contains a fragment including nucleotides 6909 to 7437 of the CaMV.
Fragment B: $DOG^{R}1$ gene from yeast which was isolated as BamHI/SalI fragment from plasmid pGEMT- $DOG^{R}1$ (cf. Figure 3).
Fragment C (192 bp): Contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-Plasmids pTiACH5.
Fragments A to C were cloned via EcoRI/HindIII into the binary vector BIN19 (Bevan (1984) Nucl. Acid Res. 12, 8711).

Figure 5

Detection of the $DOG^{R}1$ gene by PCR amplification in genomic DNA of the transgenic tobacco plants.
Lanes 1 and 14, DNA length standard; lanes 2-10, independent transformants; lane 11, untransformed control plant; lane 12, positive control (plasmid pGEMT-$DOG^{R}1$); lane 13, water control.

Figure 6

Detection of the expression of the chimeric $DOG^{R}1$ gene in 2-DOG resistant tobacco plants by RNA analysis.
Northern analysis of transgenic tobacco plants of the line 35S-DOG. Whole RNA was isolated from tobacco leaves, separated by gel electrophoresis and transferred onto a nylon membrane. Hybridization was performed with a radioactively labeled coding region of the $DOG^{R}1$ gene. 20 µg RNA were applied per lane. Lanes 1-9, independently transformed plants of the line 35S-DOG; lane 10, untransformed control.

Figure 7

Detection of the $DOG^{R}1$ gene mediated resistance in the progeny of transgenic potato plants.
Seeds of Nicotiana tabacum Var. Samsun NN were sterilized and placed on 0.05% 2-DOG containing MS medium. A: four weeks old seedlings of an untransformed plant; B: four weeks old seedlings of a plant expressing the $DOG^{R}1$ gene under the control of the 35S promoter.

Figure 8

Detection of the $DOG^{R}1$ gene mediated resistance in shoots of transgenic potato plants.
Shoots of potato plants Solanum tuberosum var. Solara were placed on 0.05% 2-DOG containing MS medium. A: five weeks old shoot of an untransformed plant; B: five weeks old shoot of a plant expressing the $DOG^{R}1$ gene under the control of the 35S promoter.

Figure 9

Plant expression cassette for the over-expression of the $DOG^{R}1$ gene from yeast (Saccharomyces cerevisiae strain 288C) in transgenic pea plants.
Fragment A (529 bp): contains the 35S promoter of the Cauliflower Mosaic Virus (CaMV). It contains a fragment comprising nucleotides 6909 to 7437 of the CaMV.

Fragment B (73 bp): untranslated translation enhancer from Tobacco Mosaic Virus U1 (Gallie (1987) Nucl. Acids Res. 15, 8693),
Fragment C: $DOG^{R}1$ gene from yeast,
Fragment D (192 bp): contains the polyadenylation signal of gene 3 of the T-DNA of the Ti plasmid pTiACH5,
Fragments A to 4 were cloned into the binary vector pGPTV (Becker (1992) PMB 20, 1195) via EcoRI/HindIII.

Figure 10

Detection of the $DOG^{R}1$ gene mediated resistance in pea plants.
Calli of pea plants Pisum sativum were placed on 0.075% 2-DOG containing B5 medium. A: calllus of untransformed plant on 0.075% 2-DOG; B: callus of a plant expressing the $DOG^{R}1$ gene under the control of the 35S promoter on 0.075% 2-DOG.] end (B)

The methods used in the examples:

1. General cloning methods

Cloning methods such as: restriction cleavage, DNA isolation, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids on nitrocellulose and nylon membranes, linking of DNA fragments, transformation of E. coli cells, culturing bacteria, sequence analysis of recombinant DNA were carried out as described in Sambrook (Cold Spring Harbor Laboratory Press (1989); ISBN 0-87969-309-6). Agrobacterium tumefaciens was transformed according to the method described by Höfgen and Willmitzer (Nucl. Acid Res. (1988) 16, 9877). The Agrobacteria were grown in YEB medium (Vervliet, Gen. Virol. (1975) 26, 33).

2. Bacterial strains

E. coli (XL-1 Blue) bacteria were obtained from Stratagene. The Agrobacterium strain (C58C1 with the plasmid pGV 3850kan) used for plant transformation was described by Deblaere (1985, Nucl. Acid Res. 13, 4777).